PHAGE DISPLAY SELECTION OF ANTI FUNGAL PEPTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/195,785, filed April 10, 2000, which is herein incorporated by reference in its entirety for all purposes.

BACKGROUND

This invention relates to the use of phage display technology to identify peptides that bind to pathogenic fungi and more particularly to pathogenic fungi of the genus *Phytophthora*. Random peptide phage display libraries are constructed using degenerate oligonucleotides. Phage expressing the peptides on their surface are contacted with fungi at different life stages and those phage that bind are isolated, amplified and the peptides identified. Once identified, peptides can be screened for anti fungal activity and used to identify and characterize binding sites on fungi.

Phytophthora is an economically important disease causing organism in the United States causing large losses in many agronomically important crop species. *Phytophthora sojae* is the second most important pathogen of soybeans in the United States. (Doupnik, *Plant Dis.* 77:1170-1171, 1993). *Phytophthora capsici* has a broad host range and most notably limits production of high-value, solanaceous vegetable crops. Control of these pathogens is particularly difficult, often requiring treatment of entire fields with biocidal compounds. Although effective, increasing concern about the environmental and economic costs of such treatments require the need for alternative control methods.

Phytophthora species are obligate parasites adapted to long-term survival in soil in the absence of host plants. Oospores or chlamydospores exist in low densities in the soil and enable survival of the pathogen. In the presence of a susceptible plant, the pathogen progresses rapidly through a series of finely tuned developmental steps that produce cycles of infection and disease. Pathogen development from oospores or chlamydospores through zoospore release, encystment, germination and infection appear straight-forward at first glance. Yet, the procession of life stages is finely tuned to environmental signals, particularly those signals coming from a host plant.

Zoospores are the life-stage of greatest importance for dispersal to root infection sites. A major susceptible site is located just behind the apical meristem of the root where cells are elongating. Exudates released from elongating cells serve as signals that direct chemotactic movement of zoospores toward the site (Carlile, in *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*, Erwin et al., eds., APS Press, 1983; Deacon and

Donaldson, *Mycol. Res.* 97:1153-1171, 1993). The zoospore chemotactic response varies with the composition of root exudates and is species specific. For example, zoospores of *P. capsici, P. cactorum*, and other species are attracted to an array of sugars and amino acids (Hickman, *Phytopathology*, 60:1128-1135, 1970; Khew and Zentmyer, *Phytopathology*, 63:1511-1517, 1973), but zoospores of *P. sojae* are attracted to specific isoflavonoid compounds (Morris et al., *Plant Physiol.*, 117:1171-1178, 1998). Although the precise mechanism of chemoattraction is not known, Deacon and Donaldson (*Mycol. Res.*, 97:1153-1171, 1993) and Carlile (in *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*, Erwin et al., eds., APS Press, 1983) summarized experiments that suggested the involvement of chemoreceptors on the zoospore surface.

Zoospores encyst as they approach the root surface in response to environmental signals. Encystment of zoospores of *P. palmivora* and other *Phytophthora* species, for example, can be influenced by local calcium ion concentrations (Griffith et al., *Arch. Microbiol.*, 149:565-571, 1988; Warburton and Deacon, *Fungal Genetics Biol.*, 25:54-62, 1998). Encystment can also be induced by high concentration of chemoattractants or by root cell wall components. For example, zoospores of *P. sojae* encyst in the presence of high concentrations of soybean isoflavonoid compounds (Morris and Ward, *Physiol. Mol. Plant Pathol.*, 40:17-22, 1992). In contrast, zoospores of *Pythium aphanidermatum* encysted when in contact with fucosyl and galactosyl residues from cell surfaces of cress roots (Longman and Callow, *Physiol. Mol. Plant Pathol.*, 30:139-150, 1987; Estrada-Garcia et al., *J. Exp. Bot.* 41:693-699, 1990). Deacon and Donaldson (*Mycol. Res.*, 97:1153-1171, 1993) noted that encystment in the presence of high concentrations of attractants would be deleterious to infection potential, and thus selected against over time. They suggested, however, that attractants at the root surface may be sufficiently concentrated to predispose zoospores to encyst after contact with root surface residues.

When in contact with a root, zoospores encyst with a specific orientation so that a germ tube emerges toward the root. If zoospores encyst before contact with the root, the germ tubes will emerge in any orientation and must re-orient in order to locate the root and infect the plant. Cell surface receptors on the germ tube are thought to be involved in this root-orientation process. Morris et al. (*Plant Physiol.*, 117:1171-1178, 1998), for example, demonstrated an oriented response of *P. sojae* germling growth to low, nontoxic concentrations of isoflavonoid compounds derived from soybeans. Zentmyer (*Science*, 133:1595-1596, 1961) reported hyphal orientation of *P. cinnamomi* toward host roots, but the nature of the attractant compound(s) was not defined.

After infection, hyphae grow through plant tissue intercelluarly and /or intracellularly depending on the species of pathogen (Stössel et al., *Can. J. Bot.*, 58:2594-

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2601, 1980; Coffey and Wilson, in *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*, Erwin et al., eds., APS Press, 1983; Enkerli et al., *Can. J. Bot.*, 75:1493-1508, 1997; Hardham and Mitchell, *Fungal Gen. Biol.*, 24:252-284, 1998; Murdoch and Hardham, *Protoplasma*, 201:180-193, 1998). Haustoria are formed by some *Phytophthora* species, including *P. infestans* (Coffey and Wilson, in *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*, Erwin et al., eds., APS Press, 1983), *P. capsici* (Jones et al., *Phytopathology*, 64:1084-1090, 1974), and *P. sojae* (Stössel et al., *Can. J. Bot.*, 58:2594-2601, 1980). Both hyphae and haustoria establish close contact with host cell walls and membranes. Presumably, cell surface receptors are important in sensing plant signals, although direct evidence is lacking. Indirect evidence of cell surface receptors comes from observations such as the occurrence of vesicles in the distal portion of haustoria (Coffey and Wilson, in *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*, Erwin et al., eds., APS Press, 1983). Heath (*Can. J. Bot.*, 73(Suppl.):S131-S139, 1995) discussed the possible events of fungal hyphal tip growth involving communication with the surrounding environment via ion channel and vesicle functions.

As discussed above, evidence points to the prominence of cell surface receptors in triggering behavioral and developmental steps of *Phytophthora*. Cells surface receptors, therefore, may provide a means for disrupting pathogen development and so infectivity. Delay or disruption of development can have a substantial impact, since zoospores have only a limited time to locate, contact, and penetrate an infection site that is effectively moving with the growing root tip. This time limitation results from the changing susceptibility of the root tissues, since as the tissues in the elongation region mature, they become significantly less susceptible to infection (English and Mitchell, *Phytopathology*, 78:1478-1483, 1988).

"Fusion phage" are filamentous bacteriophage vectors in which foreign peptides and proteins are cloned into a phage coat gene and displayed as part of a phage coat protein. The commonly used coat genes for the production of fusion phage are the pVIII gene and the pIII gene. About 3900 copies of pVIII make up the major portion of the tubular virion protein coat. Each pVIII coat protein lies at a shallow angle to the long axis of the virion, with its C-terminus buried in the interior close to the DNA and its N-terminus exposed to the external environment. Five copies of the pIII coat protein are located at the terminal end of each virion and are involved in attachment of the phage to pili of *E. coli* and for virus reassembly after infection and replication. Peptides displayed as part of pVIII are constrained in the matrix of their display on the virion coat. In contrast, peptides displayed as part of pIII are more flexible due to the terminal position of the pIII proteins. Specific phage can be constructed to display peptides of six to 15 amino

acids in length. Insertion of random or degenerate oligonucleotides into the coat protein genes allows the production of phage displayed random peptide libraries. A typical display library contains 10 to 100 copies of as many as 10^8 random sequence peptides. Thus, phage display is useful for screening for rare peptides with desired binding characteristics.

Phage-displayed random peptide libraries have been used for isolating ligands to cell surface receptors on mammalian cells. For example, peptides have been isolated from phage-displayed libraries that bind the transmembrane integrin glycoproteins involved in cell-extracellular matrix and cell-cell interactions (O'Neil et al, *Proteins*, 14:509-515, 1992; Smith et al., *J. Biol. Chem.*, 269:32788-32795, 1994; Healy et al., *Biochemistry*, 34:3948-3955, 1995). The phage displayed peptides specifically blocked cell adhesion to defined extracellular molecules and other cells (Koivunen et al., *J. Biol. Chem.*, 268:20205-20210, 1993; Koivunen et al., *J. Cell Biol.*, 124:373-380, 1994; Healy et al., *Biochemistry*, 34:3948-3955, 1995; Pasqualini et al., *Nature Biotech.*, 15: 542-547, 1997). Phage-displayed random peptide libraries have also been used to select peptides that distinguish between brain and kidney tissue (Pasqualini and Ruoslahti, *Nature*, 380:364-366, 1996). *In vivo,* affinity-selection of phage-displayed random peptides has also been used to select peptides that bind selectively to endothelial cells of blood vessels of specific tumor tissues (Pasqualini et al., *Nature Biotech.*, 15: 542-547, 1997). When these peptides were fused to an anti-cancer drug and injected into tumor-bearing mice, the peptides successfully targeted the drug to tumor blood vessels and deterred progressive tumor development (Arap et al., *Science*, 279:377-380, 1998).

Phage-display methods have been applied to plant pathogens in only very limited circumstances. Phage display methods have been used almost exclusively to identify antibodies for plant virus diagnosis (Susi et al., *Phytopathology*, 88:230-233, 1998; Ziegler et al., *Phytopathology*, 88:1302-1305, 1998; Griep et al., *J. Plant Pathol.*, 105:147-156 1999; Toth et al., *Phytopathology*, 89:1015-1021, 1999). Phage display was used in a single instance to select antibodies with affinity to surface-exposed epitopes on germlings and spores of *Phytophthora infestans* (Gough et al., *J. Immunol. Methods*, 228:97-108, 1999). Isolated phage-displayed, single-chain variable fragment (Fv) antibody fragments were not assessed for their potential to influence spore or germling behavior. Antibodies were tested for their antifungal activity with sporangia, but were found to have no detectable antifungal activity.

What is needed, therefore, is a rapid and efficient method for screening peptides for specific binding to plant pathogens. Once identified, the peptides can be further evaluated for their ability to prevent infection of plants by the pathogen, and suitable peptides can be

applied directly to the plant, used to treat the soil or, alternatively, sequences encoding the peptides can be introduced in the plants to confer immunity against the pathogen. In this manner, economical and environmentally safe and effective methods of controlling plant pathogens can be developed. The present invention meets this need.

SUMMARY

Among the several aspects of the present invention, therefore, is to provide a method for identifying peptides having an affinity for the surface of a plant pathogen comprising, constructing a library of random peptides by providing degenerate oligonucleotides encoding peptides; inserting the oligonucleotides into an appropriate vector that expresses the encoded peptides on its surface and is capable of transfecting a host cell; and transfecting an appropriate host cell with the vector to amplify the vector in a infectious form to create a library of peptides on the vector. The vector expressing the peptide library is then contacted with a target pathogen and allowed to bind to the pathogen. Unbound vector is removed and vector that has bound to the pathogen eluted. The eluted vector is then amplified in a suitable host cell and the inserted oligonucleotides isolated. The oligonucleotides are then sequenced by any suitable method and the amino acid sequence of the peptides deduced from the sequence of the oligonucleotides.

Another aspect of the invention provides an antifungal composition comprising at least one peptide selected from the group consisting of ADPPRTVST (SEQ ID NO: 7), ADRPSMSPT (SEQ ID NO: 8), ADITDPMGA (SEQ ID NO: 20), AVGTHTPDS (SEQ ID NO: 21), AVSPNVHDG (SEQ ID NO: 22), LTRCLVSTEMAARRP (SEQ ID NO: 24), EFRKNYPSAAPLIPR (SEQ ID NO: 31), LFXCYPPCTYSYCLS (SEQ ID NO: 33), and AAPDLQDAM (SEQ ID NO: 4).

Still another aspect of the present invention is a recombinant nucleotide comprising a sequence encoding a peptide selected from the group consisting of ADPPRTVST (SEQ ID NO: 7), ADRPSMSPT (SEQ ID NO: 8), ADITDPMGA (SEQ ID NO: 20), AVGTHTPDS (SEQ ID NO: 21), AVSPNVHDG (SEQ ID NO: 22), LTRCLVSTEMAARRP (SEQ ID NO: 24), EFRKNYPSAAPLIPR (SEQ ID NO: 31), LFXCYPPCTYSYCLS (SEQ ID NO: 33), and AAPDLQDAM (SEQ ID NO: 4).

Yet another aspect of the present invention is a recombinant vector comprising a nucleotide sequence encoding a peptide selected from the group consisting of ADPPRTVST (SEQ ID NO: 7), ADRPSMSPT (SEQ ID NO: 8), ADITDPMGA (SEQ ID NO: 20), AVGTHTPDS (SEQ ID NO: 21), AVSPNVHDG (SEQ ID NO: 22), LTRCLVSTEMAARRP (SEQ ID NO: 24), EFRKNYPSAAPLIPR (SEQ ID NO: 31), LFXCYPPCTYSYCLS (SEQ ID NO: 33), and AAPDLQDAM (SEQ ID NO: 4).

A further aspect of the present invention is a cell transformed with a vector comprising a nucleotide sequence encoding a peptide selected from the group consisting of ADPPRTVST (SEQ ID NO: 7), ADRPSMSPT (SEQ ID NO: 8), ADITDPMGA (SEQ ID NO: 20), AVGTHTPDS (SEQ ID NO: 21), AVSPNVHDG (SEQ ID NO: 22), LTRCLVSTEMAARRP (SEQ ID NO: 24), EFRKNYPSAAPLIPR (SEQ ID NO: 31), LFXCYPPCTYSYCLS (SEQ ID NO: 33), and AAPDLQDAM (SEQ ID NO: 4).

In still another aspect is provided an expression cassette comprising as operatively linked components, a promoter; a nucleotide sequence encoding a peptide selected from the group consisting of ADPPRTVST (SEQ ID NO: 7), ADRPSMSPT (SEQ ID NO: 8), ADITDPMGA (SEQ ID NO: 20), AVGTHTPDS (SEQ ID NO: 21), AVSPNVHDG (SEQ ID NO: 22), LTRCLVSTEMAARRP (SEQ ID NO: 24), EFRKNYPSAAPLIPR (SEQ ID NO: 31), LFXCYPPCTYSYCLS (SEQ ID NO: 33), and AAPDLQDAM (SEQ ID NO: 4); and a transcription termination signal sequence.

An additional aspect provides, a recombinant plant comprising an expression cassette comprising a promoter; a nucleotide sequence encoding a peptide selected from the group consisting of ADPPRTVST (SEQ ID NO: 7), ADRPSMSPT (SEQ ID NO: 8), ADITDPMGA (SEQ ID NO: 20), AVGTHTPDS (SEQ ID NO: 21), AVSPNVHDG (SEQ ID NO: 22), LTRCLVSTEMAARRP (SEQ ID NO: 24), EFRKNYPSAAPLIPR (SEQ ID NO: 31), LFXCYPPCTYSYCLS (SEQ ID NO: 33), and AAPDLQDAM (SEQ ID NO: 4); and a transcription termination signal sequence.

Another aspect provides a method for characterization of peptides having an affinity for the surface of plant pathogens comprising providing a library of random peptides made by providing degenerate oligonucleotides encoding peptides; inserting the oligonucleotides into an appropriate vector that expresses the peptides on its surface and is capable of transfecting a host cell; and transfecting an appropriate host cell with the vector to amplify the vector in an infectious form to create a library of peptides on the vector. The vector expressing the peptide library is then contacted with a plant pathogen of interest and the vector allowed to bind to the pathogen. After binding, the unbound vector is removed and the bound vector eluted from the pathogen. The eluted vector is amplified in a suitable host cell and the inserted oligonucleotides in the eluted vectors isolated. The peptides encoded by the oligonucleotides are then produced, contacted with plant pathogens of interest, and the effect on infectivity observed. In one embodiment, the isolated oligonucleotides are sequenced, the amino acid sequence of the peptides deduced from the nucleotide sequence, and the peptides produced by chemical synthesis. In another embodiment, peptides are produced by inserting the isolated oligonucleotides into

an expression vectors which is used to transform a suitable host cell. The transformed host cells are then maintained under conditions suitable for expression of the peptides.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims and accompanying figures where:

Figure 1 shows the organization of f8 peptide sequences into six families. On the left is the dendogram and on the right are the names and sequences of the peptides.

Figure 2 shows encystment of *P. capsici* zoospores in response to contact with the indicated f8 phage-displayed peptides at the various concentrations given. Percentage values represent the means of two experiments. The percentage encystment for the control zoospore population that contained no phage varied between 0 and 10%.

Figure 3 shows encystment of *P. capsici* zoospores in response to contact with the indicated f8 and f88-4 phage-displayed peptides at the various concentrations given.

Figure 4 shows binding of the indicated phage-displayed peptides to *P. capici* zoospsores. Values represent the mean of three experiments.

Figure 5 shows the binding specificity of the indicated phage-displayed peptides to *P. capici*. Values represent the mean of three experiments.

Figure 6 is a map of plasmid pJE-7. AOX-P is the alcohol oxidase promoter, Mat-α is the mat-alpha secretory sequence, CKX1 is the cytokinin oxidase 1 sequence, Pc87 is an exemplary peptide of the present invention, and AOX-TT is the alcohol oxidase termination sequence. Below the map is the double stranded sequence encoding Pc87 showing the (+) strand (5' AG CTA GCA GAT AGA CCA TCA ATG TCA CCA ACA TAG T 3', SEQ ID NO: 46) and the (-) strand (5' CT AGA CTA TGT TGG TGA CAT TGA TGG TCT ATC TGC T 3', SEQ ID NO: 47).

Figure 7 shows the amino acid sequence (SEQ ID NO: 48) of the exemplary insert contained in pJE-7 where the underlined nucleotides are the mat-alpha secretory sequence (cleavage by the *Kex*2 enzyme occurs after the last underlined arginine), followed by the cytokinin oxidiase 1 sequence. The two double-underlined amino acids (KL) were added to aid in construction of the fusion protein. The amino acid sequence of the exemplary peptide Pc87 is shown in bold type.

DEFINITIONS

"Secretion sequence" means a sequence that directs newly synthesized secretory or membrane proteins to and through membranes of the endoplasmic reticulum, or from the

cytoplasm to the periplasm across the inner membrane of bacteria, or from the matrix of mitochondria into the inner space, or from the stroma of chloroplasts into the thylakoid. Fusion of such a sequence to a gene that is to be expressed in a heterologous host ensures secretion of the recombinant protein from the host cell.

"Germling" means a newly germinated cyst (5-8 hr post germination) that bears an emergent germ tube.

"TBS" means Tris-buffered saline (50 mM Tris-HCl, pH 7.5, 150 mM NaCl).

A "recombinant polynucleotide" means a polynucleotide that is free of one or both of the nucleotide sequences which flank the polynucleotide in the naturally-occurring genome of the organism from which the polynucleotide is derived. The term includes, for example, a polynucleotide or fragment thereof that is incorporated into a vector or expression cassette; into an autonomously replicating plasmid or virus; into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule independent of other polynucleotides. It also includes a recombinant polynucleotide that is part of a hybrid polynucleotide, for example, one encoding a polypeptide sequence.

"IPTG" is isopropylthiogalactoside.

"TU" means transducing unit.

"NAP buffer" is 80 mM NaCl, 50 mM $NH_4H_2PO_4$, pH adjusted to 7.0 with NH_4OH.

"NZY-Tc" is a bacterial growth medium containing 1% NZ amine A (a typtone-type medium; Humko-Sheffield Chemical, Norwich, NY), 0.5% yeast extract, 0.5% NaCl, pH 7.0 adjusted with NaOH.

"PCR" means polymerase chain reaction.

As used herein "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric (2 or more monomers) form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Although nucleotides are usually joined by phosphodiester linkages, the term also includes polymeric nucleotides containing neutral amide backbone linkages composed of aminoethyl glycine units. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, labels, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), those containing pendant moieties, such as, for example, proteins (including for e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals,

radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide. Polynucleotides include both sense and antisense strands.

"Sequence" means the linear order in which monomers occur in a polymer, for example, the order of amino acids in a polypeptide or the order of nucleotides in a polynucleotide.

"Peptide" and "Protein" are used interchangeably and mean a compound that consists of two or more amino acids that are linked by means of peptide bonds.

"Recombinant protein" means that the protein, whether comprising a native or mutant primary amino acid sequence, is obtained by expression of a gene carried by a recombinant DNA molecule in a cell other than the cell in which that gene and/or protein is naturally found. In other words, the gene is heterologous to the host in which it is expressed. It should be noted that any alteration of a gene, including the addition of a polynucleotide encoding an affinity purification moiety, makes that gene unnatural for the purposes of this definition, and thus that gene cannot be "naturally' found in any cell.

A "non-immunoglobulin peptide" means a peptide which is not an immunoglobulin, a recognized region of an immunoglobulin, or contains a region of an immunoglobulin. For example, a single chain variable region of an immunoglobulin would be excluded from this definition.

"Substantially pure" or "substantially purified" means that the substance is free from other contaminating proteins, nucleic acids, and other biologicals derived from the original source organism. Purity may be assayed by standard methods, and will ordinarily be at least about 40% pure, more ordinarily at least about 50% pure, generally at least about 60% pure, more generally at least about 70% pure, often at least about 75% pure, more often at least about 80% pure, typically at least about 85% pure, more typically at least about 90% pure, preferably at least about 95% pure, more preferably at least about 98% pure, and in even more preferred embodiments, at least 99% pure. The analysis may be weight or molar percentages, evaluated, e.g., by gel staining, spectrophotometry, or terminus labeling etc.

DETAILED DESCRIPTION

The following detailed description is provided to aid those skilled in the art in practicing the present invention. Even so, this detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

All publications, patents, patent applications, databases and other references cited in this application are herein incorporated by reference in their entirety as if each individual publication, patent, patent application, database or other reference were specifically and individually indicated to be incorporated by reference.

In one embodiment peptide libraries are constructed by the insertion of nucleic acid sequences encoding peptides of six to 15 amino acids in length into suitable vectors, although sequences encoding longer peptides can be used. The peptides encoded by the nucleotides can be completely random in nature or can be constrained in their composition to meet structural or functional requirements. For example, and without limitation, a cysteine bridge can be inserted into the peptide. In one embodiment, the nucleic acid sequence does not encode an immunoglobulin (antibody) or a recognized immunoglobulin region such as a variable region. Any vector which will express the inserted oligonucleotides can be used. Preferably a vector is used which will result in expression of the peptide library on the surface of a cell or virus or on the surface of an intracellular compartment or organelle of a cell or virus. In this manner, the expressed peptides will be available to interact with potential target molecules or cells that come in contact with the surface containing the peptides. As will be apparent to one of ordinary skill in the art, if the peptides are expressed on the surface of intracellular compartments or organelles, the potential target must also reside intracellularly or the organelle or intracellular compartment must be exposed to the external environment by, for example, lysis of the cell.

Methods of producing oligonucleotides and inserting them into vectors are well known to those of ordinary skill in the art and will only be briefly reviewed herein. Most commonly, oligonucleotides are synthesized on a solid support using the phosphite triester method of Beaucage and Caruthers (*Tetrahedron Lett.* 22:1859-1862, 1981; also see, U.S. Patent Nos. 4,973,679 and 4,458,066). Numerous solid supports are available including controlled pore glass beads, polystyrene copolymers, silica gel and cellulose paper. The preparation of an oligonucleotide begins with the linkage of the 3'-hydroxyl group of the first nucleoside to the solid support. Solid supports containing nucleotides are available from commercial sources. The oligonucleotide is synthesized from the 3' to 5' direction and the chain is elongated by nucleophilic attack of the 5'-hydroxyl of the immobilized oligonucleotide on the activated 3' phosphate or phosorphramidite of a soluble 5'-protected building block. The intermediate dinucleoside phosphite formed must next be oxidized to the more stable phosphate before chain extension. The process is repeated until the desired number of nucleotides has been added. Automated devices are

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commercially available for the synthesis of oligonucleotides. In addition, numerous commercial vendors provide custom oligonucleotide synthesis services.

Any vector system capable of expressing the peptides of the peptide library may be used in the practice of the present invention and numerous vector systems are known in the art (See e.g., Wilson and Findlay, *Can. J. Microbiol.*, 44:313-329, 1998). When the peptide is displayed as part of pVIII, suitable phage systems include type 8, type 88, and type 8+8. When pIII is utilized suitable phage systems include type 3, type 33 and type 3+3. When the peptide is inserted into pVI, suitable phage systems included type 6, type 66 and type 6+6. In addition, phage T7 and phage 8 vector systems can be used. In one preferred embodiment, the peptides of the library are expressed fused to a coat protein of a filamentous bacteriophage so that the peptides are expressed on the surface of the virion and so are available to interact with target molecules or cell surface receptors. In one preferred embodiment, the f8-1 library is used in which random 8-mer peptides are fused to the pVIII coat protein. In another preferred embodiment, the f88-4 library is used in which random 15-mer peptides are fused to the pVIII coat protein. Phage in the f88-4 library display peptides without bias toward the occurrence of any amino acid. Phage in the f8-1 library are unbiased with the exception of alanine at the first position and one of four residues at the second position. All other positions are randomly occupied by any amino acid.

Methods for production of the f8-1 phage-displayed peptide library have been described previously (*See*, Petrenko et al., *Prot. Engineering*, 9:797-801, 1996 and references cited therein). The library displays foreign peptides on every copy of the 3900 copies of major coat protein pVIII. Peptide expression need not be induced by IPTG. The degenerate oligonucleotide used for the 8-amino acid insert is: GCA GNN $(NNN)_7$, where N is any nucleotide. Therefore, the first amino acid is an alanine (A) and the second amino acid is a valine (V), alanine (A), aspartate (D), glutamate (E) or glycine (G). The remainder of the amino acids in the peptide are completely randomized.

Likewise, methods for the production of the f88-4 phage-displayed peptide library have also been previously described (Zhong et al., *J. Biol. Chem.* 269:24183-24188, 1994; Smith and Scott, *Methods in Enzymology*, 217:228-257, 1993; Smith, *Gene*, 128:1-2, 1993 and references cited therein). This library displays 15-amino acid foreign peptides on 150 to 300 copies of major coat protein pVIII. The remainder of the 3900 copies of the pVIII subunits are derived from the wild type pVIII. The phage genome thus bears two pVIII genes encoding two different types of pVIII molecules. One pVIII is the recombinant displaying the foreign 15-mer peptide, while the other is the wild-type pVIII normally present on the phage. Expression of the recombinant pVIII gene is driven by the IPTG

inducible *tac* promoter/operator. Because of the presence of two pVIII genes, the f88 virion consists of a mosaic pattern of wild-type and recombinant pVIII subunits.

The oligonucleotide sequence used for the 15-mer amino acid inserts is $(NNK)_{15}$, where N is A, T, C, or G and K designates G or T. Thus, the region surrounding the 15-mer insert is:
LVPMLSFA$(X)_{15}$PAEGDDPAKA (SEQ ID NO: 1), where X is any amino acid encoded by the codon NNK.

The phage particles can be used to screen the random peptides expressed on the virion for their ability to bind to compounds and cells of interest. In one preferred embodiment, the phage-displayed peptide library is used to screen for peptides that bind to plant pathogens. In another preferred embodiment the peptides are screened for their ability to bind to pathogenic fungi. In still another preferred embodiment, phage-displayed peptides are screened for their ability to bind to members of the genus *Phytophthora*. When examining pathogens with more than a single life stage, it is preferable that each life stage be examined, since significant differences in the number, types and affinity of binding sites can occur with changes in developmental stages.

For example, when examining members of the genus *Phytophthora*, approximately 10^5 to 10^6 organisms are mixed with approximately 10^8 to 10^9 phage-displayed peptides and incubated for a time sufficient to allow binding. It will be apparent to those of ordinary skill in the art that depending on factors such as the species of pathogen, the phage and the peptide, that other concentrations of organisms and displayed peptides can be used within the scope of the present invention. In some cases, it may be desirable to pre-incubate the displayed peptides with other life stages of the same organism in order to identify those peptides that bind only to a specific life stage. After incubation, the organism is subject to multiple washes in order to remove unbound and weakly bound peptides. In the case of *Phytophthora* zoospores, washing is done using a solution of approximately 50 mM LiCl. After washing, bound phage-displayed peptides are eluted, preferrably at low pH, and the eluted phage amplified in a suitable host. In one embodiment, the host is starved K91 *E. coli*. Methods for the amplification of bacteriophage in *E. coli* are well known in the art and can be found, for example, in Smith and Scott, *Methods in Enzymology*, 217:228-257, 1993; Ausubel et al. eds., *Short Protocols in Molecular Biology*, 2nd ed., Wiley & Sons, 1995; and Sambrook et al., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, 1989. In one embodiment, the screening procedure is repeated at least once in order to enrich high-affinity phage displayed peptides. In another embodiment, the screening process is repeated three times.

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Once phage displayed high affinity peptides are identified, the phage are amplified, preferrably in *E. coli*, and the phage DNA isolated using standard methods such as those found in, for example Smith and Scott, *Methods in Enzymology*, 217:228-257, 1993; Ausubel et al. eds., *Short Protocols in Molecular Biology*, 2nd ed., Wiley & Sons, 1995; and Sambrook et al., *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory Press, 1989. Once the phage DNA has been isolated, the inserted oligonucleotides can be cleaved from the DNA using the same restriction enzymes used to insert the oligonucleotides, and the restriction enzyme fragments separated from the remainder of the DNA. The oligonucleotides can then be sequenced using any standard method. Sequencing can be carried out by any suitable method, for example, dideoxy sequencing (Sanger et al., *Proc. Natl. Acad. Sci. USA,* 74:5463-5467, 1977), chemical sequencing (Maxam and Gilbert, *Proc. Natl. Acad. Sci. USA*, 74:560-564, 1977) or any variation thereof, including the use of automatic sequencers. In one embodiment, sequencing is accomplished using an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA). Once the sequence of the oligonucleotides is known, the amino acid sequences of the peptides encoded can be readily deduced using the genetic code.

High-affinity binding, phage displayed peptides can be further screened for their ability to alter the development, growth and/or infectivity of pathogens. In this embodiment, phage-displayed peptides are incubated with a target pathogen for a time sufficient to allow binding. Following binding, the pathogen is observed for alterations in its development or ability to infect a host. In one embodiment, approximately 200 zoospores of a member of the genus *Phytophthora* are combined with a phage-displayed peptide in distilled water in two-fold serial dilutions at constant volume in petri dishes. The range of phage concentrations can vary, but generally ranges between 1 to 10 x 10^9 virion/μl. A negative control containing no phage is included in each screening. After an incubation period of usually about 20 minutes at room temperature, the number of zoospores encysted at each phage concentration is determined. Using this method it is possible to rationally select peptides of defined character and evaluate them for species- and life stage-specific induction of receptor-mediated functional responses, such a zoospore encystment. Peptides found to interfere with the development of a pathogen can be used to prevent or limit infection of a host with the pathogen.

The present method can also be used to characterize peptide binding receptors on the surface of plant pathogens. In this embodiment, peptide displaying phage that have been labeled (test phage) are incubated with cells of different organisms and at different stages of development. The relative binding affinity of the labeled phage can then be determined by competitive binding and Scatchard analysis. In a competitive binding

analysis, a constant concentration of test phage is allowed to bind to a target pathogen and then unlabeled challenge phage is added over a range of concentrations. The challenge phage may be the same as the test phage or it may be different. The target pathogen is then washed to remove non-specifically or weakly bound phage and the amount of test phage bound is determined by measuring the amount of label present on the target cells. The degree of competition can be measured as the concentration of challenge phage required to inhibit test phage binding by 50% (IC_{50}). Results from competition assays can be use to determine changes in the number, type and affinity of cell surface receptors over time.

Within the scope of the present invention are recombinant oligonucleotides discovered by the method of the present invention encoding peptides having antifungal activity. These recombinant oligonucleotides can be used to produce recombinant polynucleotides which are commonly used as cloning or expression vectors although other uses are possible. A cloning vector is a self-replicating DNA molecule that serves to transfer a DNA segment into a host cell. The three most common types of cloning vectors are bacterial plasmids, phages, and other viruses. An expression vector is a cloning vector designed so that a coding sequence inserted at a particular site will be transcribed and translated into a protein.

Both cloning and expression vectors contain nucleotide sequences that allow the vectors to replicate in one or more suitable host cells. In cloning vectors, this sequence is generally one that enables the vector to replicate independently of the host cell chromosomes, and also includes either origins of replication or autonomously replicating sequences. Various bacterial and viral origins of replication are well known to those skilled in the art and include, but are not limited to the pBR322 plasmid origin, the 2μ plasmid origin, and the SV40, polyoma, adenovirus, VSV and BPV viral origins.

The oligonucleotide sequences of the present invention may be used to produce antifungal peptides by the use of recombinant expression vectors containing the oligonucleotide sequence. Suitable expression vectors include chromosomal, non-chromosomal and synthetic DNA sequences, for example, SV 40 derivatives; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA; and viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. In addition, any other vector that is replicable and viable in the host may be used.

The nucleotide sequence of interest may be inserted into the vector by a variety of methods. In the most common method the sequence is inserted into an appropriate restriction endonuclease site(s) using procedures commonly known to those skilled in the

art and detailed in, for example, Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, (1989) and Ausubel et al., *Short Protocols in Molecular Biology*, 2nd ed., John Wiley & Sons (1992).

In an expression vector, the sequence of interest is operably linked to a suitable expression control sequence or promoter recognized by the host cell to direct mRNA synthesis. Promoters are untranslated sequences located generally 100 to 1000 base pairs (bp) upstream from the start codon of a structural gene that regulate the transcription and translation of nucleic acid sequences under their control. Promoters are generally classified as either inducible or constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in the environment, e.g. the presence or absence of a nutrient or a change in temperature. Constitutive promoters, in contrast, maintain a relatively constant level of transcription.

A nucleic acid sequence is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operatively linked to DNA for a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked sequences are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is achieved by ligation at restriction enzyme sites. If suitable restriction sites are not available, then synthetic oligonucleotide adapters or linkers can be used as is known to those skilled in the art. Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, (1989) and Ausubel et al., *Short Protocols in Molecular Biology*, 2nd ed., John Wiley & Sons (1992).

Common promoters used in expression vectors include, but are not limited to, LTR or SV40 promoter, the *E. coli* lac or trp promoters, and the phage lambda PL promoter. Useful inducible plant promoters include heat-shock promoters (Ou-Lee et al. (1986) *Proc. Natl. Acad. Sci. USA* 83: 6815; Ainley et al. (1990) *Plant Mol. Biol.* 14: 949), a nitrate-inducible promoter derived from the spinach nitrite reductase gene (Back et al. (1991) *Plant Mol. Biol.* 17: 9), hormone-inducible promoters (Yamaguchi-Shinozaki et al. (1990) *Plant Mol. Biol.* 15: 905; Kares et al. (1990) *Plant Mol. Biol.* 15: 905), and light-inducible promoters associated with the small subunit of RuBP carboxylase and LHCP gene families (Kuhlemeier et al. (1989) *Plant Cell* 1: 471; Feinbaum et al. (1991) *Mol. Gen. Genet.* 226: 449; Weisshaar et al. (1991) *EMBO J.* 10: 1777; Lam and Chua

(1990) *Science* 248: 471; Castresana et al. (1988) *EMBO J.* 7: 1929; Schulze-Lefert et al. (1989) *EMBO J.* 8: 651). Other promoters known to control the expression of genes in prokaryotic or eukaryotic cells can be used and are known to those skilled in the art. Expression vectors may also contain a ribosome binding site for translation initiation, and a transcription terminator. The vector may also contain sequences useful for the amplification of gene expression.

Expression and cloning vectors can, and usually do, contain a selection gene or selection marker. Typically, this gene encodes a protein necessary for the survival or growth of the host cell transformed with the vector. Examples of suitable markers include dihydrofolate reductase (DHFR) or neomycin resistance for eukaryotic cells and tetracycline or ampicillin resistance for *E. coli*. Selection markers in plants include resistance to bleomycin, gentamycin, glyphosate, hygromycin, kanamycin, methotrexate, phleomycin, phosphinotricin, spectinomycin, streptomycin, sulfonamide and sulfonylureas. Maliga et al., *Methods in Plant Molecular Biology*, Cold Spring Harbor Press, 1995, p. 39.

In addition, expression vectors can also contain marker sequences operatively linked to a nucleotide sequence for a protein that encode an additional protein used as a marker. The result is a hybrid or fusion protein comprising two linked and different proteins. The marker protein can provide, for example, an immunological or enzymatic marker for the recombinant protein produced by the expression vector. Suitable markers include, but are not limited to, alkaline phosphatase (AP), myc, hemagglutinin (HA), β-glucuronidase (GUS), luciferase, and green fluorescent protein (GFP).

The polynucleotide sequences of the present invention can also be part of an expression cassette that at a minimum comprises, operably linked in the 5' to 3' direction, a regulatory sequence such as a promoter, a polynucleotide encoding a peptide of the present invention, and a transcriptional termination signal sequence functional in a host cell. The promoter can be of any of the types discussed herein, for example, a tissue specific promoter, a developmentally regulated promoter, an organelle specific promoter, a seed specific promoter, a plastid specific promoter, etc. The expression cassette can further comprise an operably linked targeting, transit, or secretion peptide coding region capable of directing transport of the protein produced. The expression cassette can also further comprise a nucleotide sequence encoding a selectable marker and/or a purification moiety.

More particularly, the present invention includes recombinant constructs comprising an isolated polynucleotide sequence encoding the antifungal peptides of the present invention. The constructs can include a vector, such as a plasmid or viral vector, into which the sequence has been inserted, either in the forward or reverse orientation.

The recombinant construct can further comprise regulatory sequences, including, for example, a promoter operatively linked to the sequence. Large numbers of suitable vectors and promoters are known to those skilled in the art and are commercially available.

A further embodiment of the present invention relates to transformed host cells containing constructs comprising the oligonucleotide sequences of the present invention. The host cell can be a higher eukaryotic cell, such as a mammalian or plant cell, or a lower eukaryotic cell such as a yeast cell, or the host can be a prokaryotic cell such as a bacterial cell. Introduction of the construct into the host cell can be accomplished by a variety of methods including calcium phosphate transfection, DEAE-dextran mediated transfection, Polybrene, protoplast fusion, liposomes, direct microinjection into the nuclei, scrape loading, and electroporation. In plants, a variety of different methods can be employed to introduce transformation/expression vectors into plant protoplasts, cells, callus tissue, leaf discs, meristems, etc., to generate transgenic plants. These methods include, for example, *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation, polyethylene glycol-mediated protoplast transformation, liposome-mediated transformation, etc. (reviewed in Potrykus (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42: 205).

Peptides produced by expression of the polynucleotides of the present invention can be obtained by transforming a host cell by any of the previously described methods, growing the host cell under appropriate conditions, inducing expression of the polynucleotide and isolating the protein(s) of interest. If the protein in retained within the host cell, the protein can be obtained by lysis of the host cells, while if the protein is a secreted protein, it can be isolated from the culture medium. Several methods are available for purification of proteins and are known to those of ordinary skill in the art. These include precipitation by, for example, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography, high performance liquid chromatography (HPLC), electrophoresis under native or denaturing conditions, isoelectric focusing, and immunoprecipitation.

Alternatively, peptides encoded by the polynucleotides of the present invention can be produced by chemical synthesis using either solid-phase peptide synthesis or by classical solution peptide synthesis also known as liquid-phase peptide synthesis. In oligomer-supported liquid phase synthesis, the growing product is attached to a large soluble polymeric group. The product from each step of the synthesis can then be separated from unreacted reactants based on the large difference in size between the

relatively large polymer-attached product and the unreacted reactants. This permits reactions to take place in homogeneous solutions, and eliminates tedious purification steps associated with traditional liquid phase synthesis. Oligomer-supported liquid phase synthesis has also been adapted to automatic liquid phase synthesis of peptides.

For solid-phase peptide synthesis, the procedure entails the sequential assembly of the appropriate amino acids into a peptide of a desired sequence while the end of the growing peptide is linked to an insoluble support. Usually, the carboxyl terminus of the peptide is linked to a polymer from which it can be liberated upon treatment with a cleavage reagent. In a common method, an amino acid is bound to a resin particle, and the peptide generated in a stepwise manner by successive additions of protected amino acids to produce a chain of amino acids. Modifications of the technique described by Merrifield are commonly used (see, e.g., Merrifield, *J. Am. Chem. Soc.* 96: 2989-93, 1964). In an automated solid-phase method, peptides are synthesized by loading the carboxy-terminal amino acid onto an organic linker (e.g., PAM, 4-oxymethylphenylacetamidomethyl), which is covalently attached to an insoluble polystyrene resin cross-linked with divinyl benzene. The terminal amine may be protected by blocking with t-butyloxycarbonyl. Hydroxyl- and carboxyl-groups are commonly protected by blocking with O-benzyl groups. Synthesis is accomplished in an automated peptide synthesizer, a number of which are commercially available. Following synthesis, the product may be removed from the resin. The blocking groups are removed typically by using hydrofluoric acid or trifluoromethyl sulfonic acid according to established methods (e.g., Bergot and McCurdy, *Applied Biosystems Bulletin*, 1987). Following cleavage and purification, a yield of approximately 60 to 70% is typically produced. Purification of the product peptides is accomplished by, for example, crystallizing the peptide from an organic solvent such as methyl-butyl ether, then dissolving in distilled water, and using dialysis (if the molecular weight of the subject peptide is greater than about 500 daltons) or reverse high-pressure liquid chromatography (e.g., using a C.sup.18 column with 0.1% trifluoroacetic acid and acetonitrile as solvents) if the molecular weight of the peptide is less than 500 daltons. Purified peptide may be lyophilized and stored in a dry state until use. Analysis of the resulting peptides may be accomplished using the common methods of analytical high pressure liquid chromatography (HPLC) and electrospray mass spectrometry (ES-MS).

In general, transgenic plants comprising cells containing polynucleotides of the present invention can be produced by any of the foregoing methods; selecting plant cells that have been transformed on a selective medium; regenerating plant cells that have been transformed to produce differentiated plants; and selecting a transformed plant that expresses the protein(s) encoded by the polynucleotides of the present invention at a

desired level. Specific methods for transforming a wide variety of dicots and obtaining transgenic plants are well documented in the literature (Gasser and Fraley, *Science* 244:1293, 1989; Fisk and Dandekar, *Scientia Horticulturae* 55:5, 1993; and the references cited therein).

Successful transformation and plant regeneration have been achieved in a variety of monocots. Specific examples are as follows: asparagus (*Asparagus officinalis*; Bytebier et al. (1987) *Proc. Natl. Acad. Sci. USA* 84: 5345); barley (*Hordeum vulgarae*; Wan and Lemaux (1994) *Plant Physiol.* 104: 37); maize (*Zea mays*; Rhodes et al. (1988) *Science* 240: 204; Gordon-Kamm et al. (1990) *Plant Cell* 2: 603; Fromm et al. (1990) *Bio/Technology* 8: 833; Koziel et al. (1993) *Bio/Technology* 11: 194); oats (*Avena sativa*; Somers et al. (1992) *Bio/Technology* 10: 1589); orchardgrass (*Dactylis glomerata*; Horn et al. (1988) *Plant Cell Rep.* 7: 469); rice (*Oryza sativa*, including indica and japonica varieties; Toriyama et al. (1988) *Bio/Technology* 6: 10; Zhang et al. (1988) *Plant Cell Rep.* 7: 379; Luo and Wu (1988) *Plant Mol. Biol. Rep.* 6: 165; Zhang and Wu (1988) *Theor. Appl. Genet.* 76: 835; Christou et al. (1991) *Bio/Technology* 9: 957); rye (*Secale cereale*; De la Pena et al. (1987) *Nature* 325: 274); sorghum (*Sorghum bicolor*; Cassas et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 11212); sugar cane (*Saccharum* spp.; Bower and Birch (1992) *Plant J.* 2: 409); tall fescue (*Festuca arundinacea*; Wang et al. (1992) *Bio/Technology* 10: 691); turfgrass (*Agrostis palustris*; Zhong et al. (1993) *Plant Cell Rep.* 13: 1); and wheat (*Triticum aestivum*; Vasil et al. (1992) *Bio/Technology* 10: 667; Weeks et al. (1993) *Plant Physiol.* 102: 1077; Becker et al. (1994) *Plant J.* 5: 299).

In one preferred embodiment, plants are transformed with recombinant polynucleotides encoding the antifungal peptides of the present invention which result in the peptides being secreted by the plant. In another preferred embodiment, the antifungal peptides are secreted by the roots of the transformed plant. Plants secreting antifungal peptides can be constructed by the above described methods using expression cassettes which incorporate a secretion sequence that directs secretion of the peptides. Alternatively, plants can be transformed with a nucleotide sequence encoding a fusion protein constructed from the antifungal peptides of the present invention and a protein which is normally secreted by the plant. For example, a fusion protein can be produced between an antifungal peptide and the cytokinin oxidase enzyme. Cytokinin oxidase is a protective enzyme that acts to degrade exogenous cytokinins that could interfere with plant growth control. By fusing the antifungal peptides to the region of the cytokinin oxidase gene controlling secretion, the antifungal peptide would be secreted by the transformed plant, thus providing protection from pathogenic fungi.

Before being used to transform plants, fusion proteins containing antifungal peptides can be screened for activity using the phage display method of the present invention. In general, a fusion protein can be construction containing, an antifungal peptide; the secretory control portion of a protein, such as cytokinin oxidase; and the pVIII or pIII phage coat protein. Phage displayed fusion proteins so constructed can then be screened using the method of the present invention to select those fusion proteins that bind to a target pathogenic fungus and result in alternations which limit pathogenicity.

EXAMPLES

The following examples are intended to provide illustrations of the application of the present invention. The following examples are not intended to completely define or otherwise limit the scope of the invention.

Example 1

Fungal Species and Zoospore Production

The fungal strains used were *P. capsici* (ATCC 15399); *P. sojae* (strain 7-6-1, race 25) (A.F. Schmitthenner, Ohio State University); and *Phytophthora parasitica*. All cultures were maintained as mycelia on lima bean agar plates (*P. sojae*) or corn meal agar plates (Difco, USA) (*P. capsici* and *P. parasitica*) at 15 °C. Mycelium copies were made by transferring plugs of mycelium (5 mm x 5 mm) to agar plates containing clarified 10% V8® vegetable juice (Campbell Soup Co., USA). Three plugs per plate were grown for three to six days at 25 °C. Sporangia production was induced in *P. capsici* by trimming the plates and incubating at 25 °C with light. After one to two days, zoospore release was induced by flooding the plates with sterile water for 20 to 30 minutes. *P. parastica* zoospore production was identical to that of *P. capsici* except that the plates were washed with sterile water for two minutes prior to incubating at 25 °C with light. Zoospore release was induced from *P. sojae* sporangia by flooding the plates four times in sterile water at 30 minute intervals. Zoospores were released within two to four hours. After their release, zoospores were filtered through four layers of cheesecloth to remove sporangial cases and mycelial fragments. A sample of the suspension was vortexed for 30 seconds to induce encystment and the cysts counted under a microscope in a hemacytometer.

Example 2

Preparation of Starved K91kan *E. coli* Cells and Titering Phage as Transducing Units

Prior to library screening, the phage were titered as tetracyclin transducing units (TU) in starved K91BluKan (kanomycin resistant) *Escherichia coli* cells, according to

published methods (Smith & Scott, *Methods in Enzymology*, 217:228-257, 1993; Yu and Smith, *Methods in Enzymology*, 267:3-27, 1996). Transducing units are an effective way of measuring the infectivity of the phage and are usually expressed as TU/ml of phage. In brief, K91BluKan cells were grown at 37°C with vigorous shaking (~170 rpm) in 20 ml superbroth (Smith and Scott, *Methods in Enzymology*, 217:228-257, 1993) to mid log phase (OD_{600} ~0.45). The cells were then incubated with gentle shaking for an additional 5 minutes to allow any sheared F pili to regenerate. The cells were centrifuged in a sterile 50 ml Oak Ridge tube at 2,200 rpm for 10 minutes in a Sorvall SS34 rotor at 4°C. The supernatant was poured off and the cells were resuspended in 20 ml of 80 mM NaCl, placed in a 125 ml culture flask and shaken gently for 45 minutes at 37°C and 70 rpm. The cells were then centrifuged as above and resuspended in 1 ml cold NAP buffer. The starved cells were stored at 4°C and remained infective for 3 to 5 days.

The phage were titered as transducing units (TU) in *E. coli* K91BluKan starved cells (prepared as above). Phage were analytically titered using TBS/gelatin as the diluent. Ten microliters of each phage dilution were deposited as a droplet on the inner wall of a 15 ml sterile disposable tube held at a 10° angle from the horizontal. Ten microliters of starved *E. coli* K91BluKan cells were added to each phage droplet and this was incubated for 10 minutes at room temperature to allow time for the phage to infect the concentrated cells. After 10 minutes, 1 ml of superbroth containing 0.2 mg/ml tetracyclin was added to the cells and incubated for 20 to 40 minutes at 37°C with shaking. For amplification of the f88-4/15 mer phage, the superbroth also contained 1 mM IPTG to induce recombinant pVIII expression. The infected cells were then spread (200 ml per plate) on Luria-Bertani (LB) plates containing 40 mg/ml tetracyclin. The plates were then incubated for ~24 hr at 37°C.

Example 3

Selection of Zoospore Binding Phage

An aliquot of 10^{11} transducing units (TU) from the f8-1 library (Petrinko et al., *Protein Engineering*, 9:797-801, 1996) was added to 10^6 freshly released *P. capsici* zoospores at room temperature in 4 ml of 50 mM LiCl and incubated for 30 minutes at room temperature with gentle agitation. The same procedure was used for the f88-4 library except in some cases *P. sojae* zoospores were used (Soj clones). The zoospores containing the bound phage were washed 10 times in 150 µl of 50 mM LiCl and centrifuged at 1000 x g for 45 seconds to remove unbound phage. After the tenth wash, the bound phage were eluted with 200 µl of elution buffer (0.1 N HCl, glycine sufficient to bring pH to 2.2, 1 mg/ml bovine serum albumin). The eluted phage were amplified by

infection of starved *E. coli* K91BluKan cells as described above. The amplified phage were then purified by precipitation with polyethylene glycol as described below, and resuspended in TBS buffer as described by Smith and Scott (*Methods in Enzymology*, 217:228-257, 1993). An aliquot of purified phage was subsequently re-applied to freshly released zoospores, as described above for a total of three affinity purifications and two amplification steps. Selective enrichment of the zoospore-binding phage was monitored by calculating the percent yield after each round of selection as described in Smith and Scott (*Methods in Enzymology*, 217:228-257, 1993). This was done by calculating the total phage (expressed as transducing units) that was applied to the zoospores and measuring the total output of phage (as transducing units) that was recovered from the zoospores and expressing the result as a percentage. The yield of phage eluted from the zoospores after each round of screening was between 10^{-4} to 10^{-5} % indicating that this procedure was successful in selecting for phage binding to the zoospores. The zoospores were intact and spherical after the washing steps, showing that little or no encystment had occurred during the selection process.

Example 4
Phage Purification

E. coli K91BluKan cells infected with phage were grown overnight in 20 ml superbroth (containing 40 mg/ml tetracyclin) at 37°C and 170 rpm. The culture was centrifuged to pellet the *E. coli* cells (containing phage) in a SS34 rotor for 10 min at 5,000 x g. The supernatant was removed and placed in a new Oak Ridge tube and PEG/NaCl (16.7% polyethylene glycol/3.3 M NaCl) was added at a rate of 150 µl per ml supernatant to precipitate the phage. The phage were precipitated overnight at 4°C and then pelleted by centrifugation in a 50 ml Oak Ridge tube at 10,000 rpm for 20 min in a SS34 rotor. The pelleted phage were resuspended in 1 ml of Tris-buffered saline (TBS). This was again re-precipitated by the addition of 150 ml PEG/NaCl and left overnight at 4°C. The phage were pelleted by centrifugation in a bench top centrifuge and the pellet was re-suspended in TBS.

Example 5
DNA Isolation, Sequencing and Analysis

DNA used for sequencing was isolated from individual phage clones according to the method of Smith and Scott (*Methods in Enzymology*, 217:228-257, 1993). Single-stranded DNA was sequenced from the 3' end using an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA) following the manufacturer's protocol.

The primer used for f8 clones was 5'-GGAGCCTTTAATTGTATCGG-3' (SEQ ID NO: 2). The primer used for f88 clones was 5'-AGT AGC AGA AGC CTG AAG A-3' (SEQ ID NO: 3).

DNA sequences were translated using the "translate" program of the ExPASy Molecular Biology Server (website http://www.expasy.ch/). Sequences were compared with nucleic acid and protein sequences stored in sequence databases (GenBank, EMBL, dbEST, SwissProt, PIR) using standard algorithms (i.e.) FASTA (Lipman and Person, *Science*, 227:1435-1441, 1985) and BLAST (Altschul et al., *J. Molecular Biol.*, 215:403-410, 1990) commands. Peptide sequences were aligned using ClustalW (Thompson et al., *Nuc. Acid Res.*, 22:4673-4680, 1994) with a PAM250 weight table and the dendogram viewed using TreeView (Page, *Computer Applic. Biosci.*, 12:357-358, 1996). The f8-mer DNA sequences obtained coded for 19 predicted peptide sequences (Table 1). The majority of the peptides contained amino acid residues that were predicted to be strong α-helical formers (i.e. Glu, Ala and Leu) and α-helical breakers (i.e. Gly and Pro). Despite the lack of a common motif, the ClustalW multiple sequence alignment program was used to cluster similar peptides in the form of a dendogram. The dendogram, constructed from the aligned peptides, indicated that the f8-mer peptide sequences could be grouped into six broad family groups as depicted in Figure 1 and Table 1. Selected sequences from the f88-4/15 mer library are shown in Table 2.

Example 6

Encystment Assay

Selected phage clones were isolated according to Smith and Scott (*Methods in Enzymology*, 217:228-257, 1993), and twice purified using polyethylene glycol as described above with the exception that phage were resuspended in distilled water instead of TBS. The virion concentration was calculated by measuring the absorbance at A_{269} (Smith and Scott, *Methods in Enzymology*, 217:228-257, 1993). Water droplets of approximately 20 μl containing about 400 freshly released zoospores were incubated with phage at two-fold serial dilutions so that they contained phage-bearing peptides at concentrations of either 1×10^{10}, 5×10^9, 2.5×10^9 or 1.25×10^9 virion/μl of droplet. A negative control received no phage and was used to monitor the amount of spontaneous encystment in the zoospore population. After a 20 minute incubation at room temperature, the number of encysted zoospores was counted using a microscope at 100X magnification. The virion concentration of phage was calculated according to Smith and Scott (*Methods in Enzymology*, 217:228-257, 1993).

The effectiveness of the f8 peptides in inducing premature encystment varied with sequence family and with phage concentration (Figure 2). At a concentration of 1 x 10^{10} virion/μl (64 μM), many peptide families were effective in inducing encystment. At a concentration of 2.5 x 10^9 virion, however, there was a 3- to 5-fold difference in the peptides that caused high levels of encystment (Pc42, Pc78, Pc87 and Pc64) and peptides causing low levels of encystment (Pc15B, Pc56 and Pc45). At a concentration of 1.25 x 10^9 (8 μM), all peptide families were minimally effective in inducing encystment. The wild-type phage also caused encystment of the *P. capsici* zoospores; however, the fraction of zoospores encysted by the selected phage was two to seven times greater than the fraction encysted by the wild-type phage in all experiments. The ability of *P. capsici* selected, phage-bearing peptides to prematurely encyst zoospores was specific for *P. capsici*. Little or no encystment was observed when *P. sojae* and *P. parastica* zoospores were incubated with phage-bearing peptides at 1 x 10^{10} virion/μl, a concentration that resulted in almost 100% encystment for *P. capsici* zoospores. Similar results were obtained with f88-4 15 mer peptides. The ability of representative 15 mer peptides to cause premature encystment in comparison to the f-8 clone Pc87 is shown in Figure 3.

Example 7
Binding Specificity

Phage-displayed peptides with high and low encystment induction abilities were compared for their ability to bind to *P. capsici* zoospores. Phage clones Pc87 and Pc45 were randomly selected as the representative clones that induced high and low levels of encystment, respectively (cf. Figure 2). Phage vector was included as a control treatment. Phage clones were amplified by *E. coli* infections and purified as described above. For each binding reaction, 5 x 10^{10} TU of phage were incubated with 200,000 *P. capsici* zoospores. The binding reaction and washes were performed as described in Example 3 for phage selection. Phage eluted from the zoospore population were titered in *E. coli* K91BluKan cells and expressed as total transducing units. A similar procedure was used to determine whether the selected phage bound to *P. capsici* cysts.

Phage vector and clones Pc45 and Pc87 bound differentially to *P. capsici* zoospores. More than 10^7 TU of phage Pc87 were eluted from zoospores after 30 minutes incubation, while only about 50,000 TU of phage Pc45 or phage vector were eluted under the same conditions (Figure 4). Moreover, binding was specific for the zoospore stage: less than 10^4 Pc87 TU were eluted from cysts—about the same background binding observed with control vector phage (Figure 5).

Example 8

Construction of Secreted Fusion Protein

Carboxy-terminal DNA fusions were constructed to encode for the peptides of interest by ligation of synthetic oligonucleotides into the restriction enzyme sites, *Hind*III and *Xba*I, of the carrier vector pJE-6. The plasmid vector pJE-6 was constructed from the *Pichia pastoris* expression construct pROM-46 derived from the plasmid pPICZ-alpha (Invitrogen, Carlsbad, CA), previously described (Cregg et al., *Bio/Technology*, 11:905-910, 1993; Rosenfeld, *Methods in Enzymology*, 306:154-169, 1999). Plasmid pROM-46 was digested with restriction endonuclease, *Hind*III, filled-in with Klenow enzyme and dNTP's, and re-ligated withT4 DNA ligase. These steps eliminated a *Hind*III restriction site present within the pPICZ-alpha plasmid sequence, and the plasmid was designated pJE-4. The sequence at the 3' end of the coding sequence was mutagenized by PCR to replace the stop codon with the restriction site *Hind*III. This plasmid was designated pJE-6. Synthetic oligonucleotides encoding for an exemplary peptide (Pc87, ADRPSMSPT, SEQ ID NO: 8), were ligated into the plasmid pJE-6, digested with *Hind*III and *Xba*I. This plasmid designated pJE-7 (Figure 6). The pJE-7 plasmid was sequenced to confirm the insert and the results are in shown in Figure 7.

CONCLUSION

In light of the detailed description of the invention and the examples presented above, it can be appreciated that the several aspects of the invention are achieved.

It is to be understood that the present invention has been described in detail by way of illustration and example in order to acquaint others skilled in the art with the invention, its principles, and its practical application. Particular formulations and processes of the present invention are not limited to the descriptions of the specific embodiments presented, but rather the descriptions and examples should be viewed in terms of the claims that follow and their equivalents. While some of the examples and descriptions above include some conclusions about the way the invention may function, the inventors do not intend to be bound by those conclusions and functions, but put them forth only as possible explanations.

It is to be further understood that the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention, and that many alternatives, modifications, and variations will be apparent to those of ordinary skill in the art in light of the foregoing examples and detailed description. Accordingly, this invention

is intended to embrace all such alternatives, modifications, and variations that fall within the spirit and scope of the following claims.

Table 1

FAMILY	CLONE	AMINO ACID SEQUENCE	SEQ ID NO
1	Pc56	AAPDLQDAM	4
2A	Pc19	ADRLNSDAG	5
	Pc36	ADRPSTTSL	6
	Pc78	ADPPRTVST	7
	Pc87	ADRPSMSPT	8
	Pc11	ADRTSNAST	9
2B	Pc76	ADKSYIPSS	10
	Pc65	AVRNPSHHS	11
	Pc44	ADPTPRGHS	12
	Pc58	ADPTRQPHS	13
3A	Pc45	AEHQNSAGP	14
	Pc14	ADARSAGAIS	15
	Pc39	ADSKNAGPM	16
	Pc53	AETKFSGSA	17
	Pc15A	ADPKGSGVT	18
3B	Pc15B	AGLTSPNDM	19
	Pc43/PC64	ADITDPMGA	20
4	PC29B	AVGTHTPDS	21
	Pc12/Pc42	AVSPNVHDG	22

Table 2

CLONE	AMINO ACID SEQUENCE	SEQ ID NO.
Cap1/18	VAAFSLVWATHLMLS	23
Cap1/12	LTRCLVSTEMAARRP	24
Cap1/9	SAPYLPYFDLLHFPI	25
Cap1/13	PSSYEASRRPEHWXF	26
Cap1/11	SATDTTLPMMTAIRS	27
Cap1/22	TRLSPMESXAMLLAP	28
Cap1/20	LLPVSPPFAPNASST	29
Cap1/24	MSNFPTSHAPCPVEI	30
Cap1/6	EFRKNYPSAAPLIPR	31
Cap1/23	PXVHGSIPLTPPLGF	32
Cap1/30	LFXCYPPCTYSYCLS	33
Cap1/1	MSNFPTSHAPCPVXI	34
Cap1/16	PEWKSSWSPCTPRCP	35
Cap1/28	AMSRWLRPRE(M/I)NAPP	36
Cap1/19	THTTFXVTVXLHEPP	37
Cap1/27	MTSPRNSQLIVPFCL	38
Cap1/7	PTLGRFNRPSCSIIV	39
Soj2-2	APQCHPHLPFDMIHV	40
Soj2-3	NHNSLPAQYLVXILR	41
Soj2-4; Soj2-6	DQPCTPSPDVSFYRS	42
Soj2-8	VAAPSHWLKPSLDCF	43
Soj2-9	NPLYKNPPPRVAMCL	44
Soj2-19	LIFRYAPPPLFLRPP	45

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